AD_____

Award Number: DAMD17-03-1-0173

TITLE: Function of Human Selenium-Binding Protein in Selenium Induced Growth Arrest and Apoptosis in Prostate Cancer Cells

PRINCIPAL INVESTIGATOR: Yuxun Wang, Ph.D.

CONTRACTING ORGANIZATION: Beth Israel Deaconess Medical Center
Boston, Massachusetts 02215

REPORT DATE: April 2004

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 2004	Annual Summary (1 Apr 2003 - 31 Mar 2004)

4. TITLE AND SUBTITLE
Function of Human Selenium-Binding Protein in Selenium Induced Growth Arrest and Apoptosis in Prostate Cancer Cells

5. FUNDING NUMBERS
DAMD17-03-1-0173

6. AUTHOR(S)
Yuxun Wang, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Beth Israel Deaconess Medical Center
Boston, Massachusetts 02215

E-Mail: ywang1@caregroup.harvard.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The proposed study required the creation of prostate cell lines that contain hSP56 transgene and it is regulated artificially. During the first year of this study, preparation of inducible mammalian expression construct of hSP56 gene and anti sense expression construct was accomplished. Prostate cancer cell line PC-3 expresses hSP56 extremely low and they are stably transfected with the above-described expression constructs. Independent clones of PC-3 cells that contain transgene of hSP56 were selected and screened for inducible expression of hSP56 by immunoblotting. Anti-sense RNA method was used to down-regulated the endogenous hSP56 in LNCaP cells and an effective interference RNA was identified for hSP56 gene. The down regulation of hSP56 in LNCaP cells was confirmed by immunoblotting as well.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Selenium, gene expression, gain-of-function, loss-of-function, cDNA, microarray	10
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

DAMD17-03-1-0173
Yuxun Wang
(revised by Nataša Debeljak)

Annual Summary (1 April 2003 - 31 March 2004)

Background

The factors regulating normal prostate epithelial development, neoplastic transformation and prostate cancer growth and spread are poorly understood. It is our long-term goal to identify those genes and gene products responsible for the regulation of prostatic epithelial differentiation and its malignant transformation, for the vast differences in pathobiology and clinical progression among individual tumors, and to identity new therapeutic targets and prognostic markers. Our laboratory has identified several genes, including selenium-binding protein (hSP56) that show differential expression between androgen-sensitive LNCaP and androgen-insensitive PC-3 prostate cancer cell lines [1]. These two prostate cell lines differ not only in sensitivity to hormone for growth but also in their aggressiveness in terms of metastasis. We have since confirmed that hSP56 is differentially expressed by Northern and Western analyses and that it is down-regulated by androgen in cultured LNCaP cells. The inhibitory effect of selenium compounds in cell proliferation was evident in LNCaP cells, which produce high levels of hSP56, however, was not observed in PC-3 cells, which express very low levels of hSP56.

Aim of the study

We hypothesize that hSP56 plays important yet unrecognized roles in the regulation of cell proliferation, and/or apoptosis, or other processes in normal prostate and prostate cancer cells in response to environmental changes in selenium availability. We initially used the name hSeBP to describe this protein, however, we have since changed it to hSP56 to be consistent with the nomenclature used by NCBI genomic database. The aim of the study is to elucidate the biological functions of hSP56 in prostate epithelial cell and cancer phenotype. This will potentially aid future development of selenium-containing small molecules as therapeutic drugs for prostate cancer. Our results will have applications in other malignancies as well because of the expression of hSP56 in other cancer cells.

Results

In the first year of the study we used the antisense RNA mediated method to suppress the expression of endogenous hSP56 gene in LNCaP cells and to study the consequence of loss of this protein. We applied two different approaches to down regulate the endogenous hSP56 in LNCaP cells. First, the hSP56 coding DNA was inserted into the pcDNA4/TO/*myc*-His vector (Invitrogen) in reverse orientation. An antisense mRNA was

transcribed from the transgene under induction. For the second type of loss-of-function study, the siRNA technique was applied. Using accepted rules [2], five siRNA hSP56 sequences were designed and synthesized. Direct transfection of siRNA oligonucleotides targeting different regions of hSP56 gene was used to examine their effectiveness in downregulation of hSP56 expression in LNCaP cells. Northern blots showed that one of the five oligonucleotides down-regulated hSP56 mRNA by 70-80 % (siRNA # 2, Figure 1).

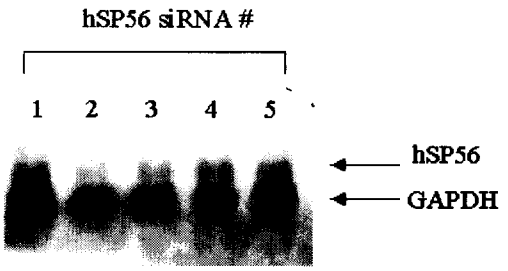

Figure 1. Northern blot shows down-regulation of hSP56 mRNA in LNCaP cells by siRNA # 2. Control GAPDH transcript is not affected.

Venkateswaran et al. showed that selenomethionine (SeMeth) inhibited the growth of LNCaP but not PC-3 cells *in vitro*, and they speculated that this difference might be due to the fact that LNCaP cells express hSP56, whereas PC-3 cells do not [3]. We grew LNCaP in the absence or presence of the siRNA and in the absence or presence of SeMeth followed by sequential cell counts. As expected, in the absence of the siRNA, SeMeth inhibited LNCaP growth in a concentration-dependent manner (Figure 2, left panel). However, down-regulating hSP56 by addition of siRNA #2 markedly reduced the growth-inhibiting action of SeMeth; the cells grew more rapidly. In contrast, addition of siRNA # 5, which had little effect on hSP56 expression assessed by northern, did not reduce the growth-inhibiting action of SeMeth (Figure 2, right panel).

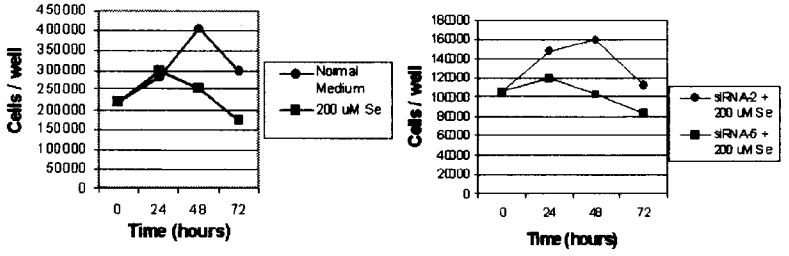

Figure 2. Effect of selenomethionine on LNCaP growth in the absence or presence of hSP56 siRNA. Left panel, growth curve in normal medium (top curve, closed circles) or in medium containing SeMeth (bottom curve, closed squares). Right panel, growth curves in medium containing SeMeth and hSP56 siRNA. Top curve, closed circles: siRNA #2 down-regulates hSP56 mRNA and blocks the growth inhibiting action of SeMeth. Bottom curve, closed squares: siRNA #5 fails to down-regulate hSP56 and does not block SeMeth growth inhibition of LNCaP.

For gain-of-function studies, we created PC-3 cell lines in which the expression of exogenous hSP56 protein is up-regulated artificially. The entire coding sequence of hSP56 gene was inserted into the mammalian expression vector pcDNA4/TO/*myc*-His vector. This vector contains the human cytomegalovirus immediate-early (CMV) promoter with tetracycline operator sites for inducible expression of hSP56 protein. The correct insertion of hSP56 DNA is confirmed before transfection into PC-3 cells. Selection of homogenous cells from single clone has been successful. The inducible expression of hSP56 in this PC-3 cell line has been observed under the induced condition by immunobloting using our hSP56 antibodies. It was clear that the cells with expression construct produced many fold more of hSP56 protein when induced by tetracycline (data not shown).

Then we carried out growth assays in the absence or presence of SeMeth similar to the assay with the LNCaP cell line. In the absence of SeMeth, the growth rates of wild type PC-3 and PC-3/hSP56 cells were nearly identical. Also, we confirmed the finding that SeMeth had no significant inhibitory action on the growth of the wild-type PC-3 cells (Figure 3, left panel). Importantly, SeMeth inhibited the growth of the PC-3/hSP56 cells markedly (Figure 3, right panel), strongly supporting the hypothesis that hSP56 plays a critical role in mediating the growth-inhibiting action of SeMeth on prostate cancer cells.

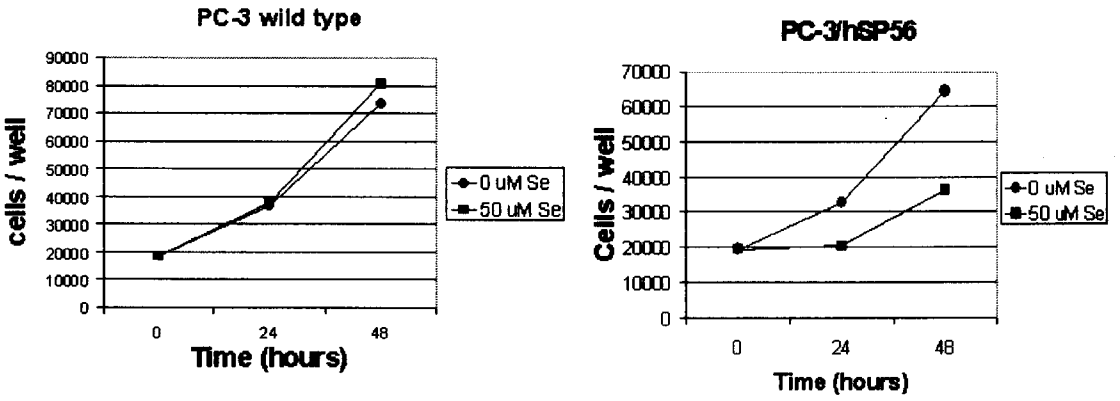

Figure 3. Effect of selenomethionine on growth of PC-3 and PC-3/hSP56 cells. Left panel, SeMeth has not effect on growht of wildtype PC-3 cells. Right panel, SeMeth markedly inhibits growth of hSP56-expressing PC-3/hSP56 cells. Closed circles, normal medium. Open circles, SeMeth-containing medium.

During this period of work, I not only prepared the necessary cells for future study of the function of hSP56 in tumor cells, but also gained training in mammalian cell culture, transfection and selection of transfected cells. An additional benefit of this study is my better understanding of inducible mammalian cell expression systems and the gene expression regulation of transgene in mammalian cells. I have accomplished the above-described tasks within the proposed time period, and have started characterizing the phenotypic changes caused by up-regulation of hSP56 in PC-3 cells and down-regulation of hSP56 in LNCaP cells.

We have also performed experiments to demonstrate that hSP56 protein is not a selenoprotein but contains covalent Se. Mammalian Se-containing proteins can be divided into two groups. The first group comprises the selenoproteins, proteins contain selenocysteine residues. The other Se-containing proteins with yet undetermined chemistry fall into the second group, sometimes designated selenium-binding proteins. Among the selenoproteins are some well-characterized enzymes such as thioredoxine reductases, glutathione peroxidases, and iodothyronine deiodinases. Selenium is incorporated into these proteins in the form of selenocysteine, and the selenocysteine residues have been experimentally determined to serve as functional components of the active enzymatic domains in some of these enzymes. The selenocysteine residues are inserted specifically into the selenoproteins through a co-translational mechanism directed by the UGA codon (normally serves as stop codon) and by a specific RNA sequence (termed selenocysteine insertion sequences, or SECIS) located in the 3' untranslated region (3'UTR) of the mRNA. The SECIS is hypothesized to form a specific secondary structure. Protein factor(s) with SECIS RNA binding activity have been purified and shown to interact with the SECIS sequence to direct the ribosome to recognize the selenocysteine insertion codon [4, 5]

Although there is nucleotide sequence with some similarity to the consensus SECIS element in the 3' untranslated region of hSP56 gene, the sequence is not functional in directing selenocysteine insertion. We tested the ability of the hSP56 3'UTR to direct the insertion of selenocysteine into the coding region of human type I deiodinase (in collaboration with Dr. Marla Berry). A mammalian expression vector containing human type I deiodinase (107-112-D10) was cleaved with Hind III and Not I to remove the functional SECIS sequence, replacing it with a 265 bp DNA fragment from the 3' UTR of hSP56 (107-112-D10/hSP563UTR). Oligonucleotide primers SECISP1 (5'-CCCAAGCTTTAGACTCCACCCTCATCACCC-3') and SECISP2 (5'-ATAGTTTAGCGGCCGCGAAGGACAGGGTTACGAGTT-3') corresponding to positions 1467-1484 and 1685-1704 of hSP56 BC009084 were synthesized and used in PCR amplification. The PCR primers were designed to include Hind III and Not I sites at their termini. High fidelity PCR reaction with primers SECISP1 and SECISP2, using hSP56-BC009084 plasmid DNA as template, generated the DNA fragment of the hSP56 3'UTR. This DNA was tested for its potential selenocysteine insertion function after being cleaved with restriction enzymes and inserted into the Hind III and Not I sites of expression construct 107-112-D10. Human type I deiodinase requires the insertion of a selenocysteine to be enzymatically active. The activity of SECIS sequence thus is assayed by measuring the type I deiodinase enzyme activity in the cellular protein extract. We did not detect significant deiodinase activity in cells transfected with our test construct DNA (Figure 4). In fact, we observed similarly low (near background) deiodinase activity in cells transfected with our testing construct and vector control construct. However, the deiodinase activity in our positive control samples was more than 30 times higher than control.

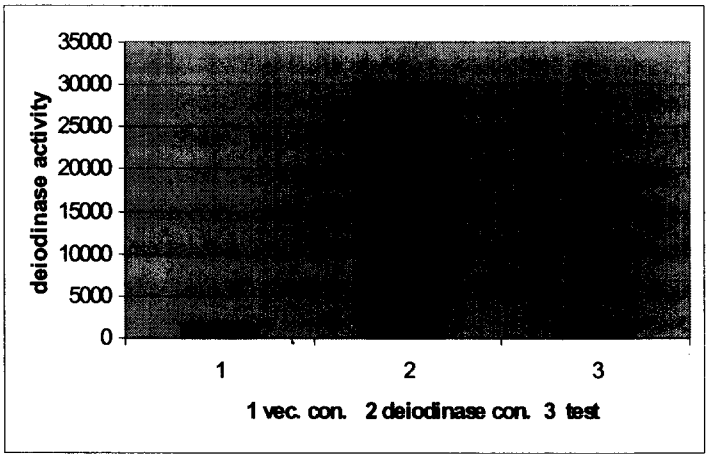

Figure 4. Deiodinase assay shows that 3' UTR of hSP56 does not contain an SECIS. 1) Negative control; 2) Positive control; 3) hSP56 3'UTR shows no activity.

Despite the lack of selenocysteine insertion, we demonstrated that selenium could be stably incorporated into endogenous hSP56 in LNCaP cells. $1X10^7$ cells were grown in the presence of 192 μCi ^{75}Se (as Na selenite) for 24 hours. Lysates were prepared and subjected to immunoprecipitation with anti-hSP56 or irrelevant antibodies followed by SDS-PAGE and autoradiography (Figure 5). Protein A agarose alone did not bind ^{75}Se-labeled hSP56 (lane 1), however, anti-hSP56 antibody immunoprecipitated ^{75}Se-hSP56, and the amount of labeled ^{75}Se-hSP56 protein detected correlated with the amount antibody added (compare lanes 2 and 3 in Figure 5). An irrelevant antibody raised against an unrelated protein did not precipitate hSP56 (lane 4). Although the ^{75}Se binding to hSP56 in LNCaP cells was detected in repeat experiments, in no experiment was the binding especially robust. This contrasts with the original labeling experiments of Bansal et al. who injected the isotope intraperitoneally into mice and allowed physiologic processing of the isotope leading to identification of the rat homologue SP56 [6]. It may be that a low rate of processing of the radioisotope by the clonal cell line LNCaP is responsible for the difference. This work is included in our manuscript, Selenium binding protein hSP56 contains covalent Se but not by selenocysteine insertion, which will be submitted to Journal of Biological Chemistry for publication.

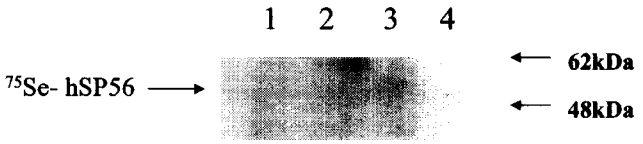

Figure 5. ^{75}Se binding to hSP56 in LNCaP cells. Immunoprecipitated with 1) protein A agarose alone (no Ab); 2) 5 μg anti-hSP56 Ab; 3) 20 μg anti-hSP56; 4) 20 μg irrelevant Ab. Note trace amount of ^{75}Se-hSP56 in lane 2 and increased amount in lane 3 corresponding to anti-hSP56 addition. No ^{75}Se-hSP56 detected in lanes 1 and 4. Molecular weight markers at right.

Key accomplishments

- Successful transfection of mammalian expression vector containing hSP56 cDNA into PC-3 cells.
- Selection of stably transfected PC-3 cells that express hSP56 protein and isolation of clones from single cell colonies.
- Demonstration of down regulation of hSP56 in LNCaP cells by siRNA technology.
- Evidence of inhibitory effect of selenium compounds in cell proliferation in LNCaP cells but not in PC-3 cells.
- Demonstrated by isotope labeling and immunoprecipitation that hSP56 contains covalent selenium.
- Shown by SECIS activity assay that hSP56 is not a selenoprotein which contains selenocysteine residue.

Other outcomes

The work on the selenium binding property of hSP56 protein was presented at two meetings.

1. American Association for Cancer Research (AACR), 95th Annual Meeting, March 27-31 2004, Orlando:
   a. Human Selenium Binding Protein Hsp56, A Putative Chemopreventive Protein, Binds Selenium By A Novel Mechanism; Yuxun Wang and Arthur J. Sytkowski (#4054)
   b. Role of Human Selenium Binding Protein 56 (hSP56) in Selenium's Growth Inhibitory on Human Prostate Carcinoma Cells; Chong Gao, Yuxun Wang, Changmin Chen, Laurie Feldman and Arthur J. Sytkowski (#1394)

2. American Society of Hematology (ASH), 45th Annual meeting, December 6-9 2003, San Diego:
   a. Selenium Binding Protein hSP56 Is Present In Abundance In Human Erythrocytes; Yuxun Wang and Arthur J. Sytkowski (#3717)

The same set of data are being prepared for future publication.

References

1. Yang, M. and A.J. Sytkowski, Differential expression and androgen regulation of the human selenium-binding protein gene hSP56 in prostate cancer cells. Cancer Research, 1998. 58(14): p. 3150-3.
2. Elbashir, S.M., J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, and T. Tuschl, Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature, 2001. 411(6836): p. 494-8.
3. Venkateswaran, V., L.H. Klotz, and N.E. Fleshner, Selenium modulation of cell proliferation and cell cycle biomarkers in human prostate carcinoma cell lines. Cancer Research, 2002. 62(9): p. 2540-5.
4. Berry, M.J., R.M. Tujebajeva, P.R. Copeland, X.M. Xu, B.A. Carlson, G.W. Martin, 3rd, S.C. Low, J.B. Mansell, E. Grundner-Culemann, J.W. Harney, D.M. Driscoll, and D.L. Hatfield, Selenocysteine incorporation directed from the 3'UTR: characterization of eukaryotic EFsec and mechanistic implications. Biofactors, 2001. 14(1-4): p. 17-24.
5. Martin, G.W., 3rd, J.W. Harney, and M.J. Berry, Selenocysteine incorporation in eukaryotes: insights into mechanism and efficiency from sequence, structure, and spacing proximity studies of the type 1 deiodinase SECIS element. RNA-A Publication of the RNA Society, 1996. 2(2): p. 171-82.
6. Bansal, M.P., C.J. Oborn, K.G. Danielson, and D. Medina, Evidence for two selenium-binding proteins distinct from glutathione peroxidase in mouse liver. Carcinogenesis, 1989. 10(3): p. 541-6.